Exhibit D

Amplified expression of the HER2/ERBB2 oncogene induces resistance to tumor necrosis factor α in NIH 3T3 cells

(gene amplification/receptor transmodulation/tumorigenesis)

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ABSTRACT Functional characterization of oncogene products that induce cellular transformation has progressed rapidly in recent years. However, less is known about the mechanism(s) by which the transformed cells may escape destruction by host immune defenses and form tumors. A recently described oncogene that has an important association with aggressive human breast carcinoma is "HER2," for human epidermal growth factor receptor 2. The oncogene has also been called *NGL* and human c-*erbB-2* (*ERBB2*). In this paper we show that amplification of HER2 oncogene expression can induce resistance of NIH 3T3 cells to the cytotoxic effects of recombinant tumor necrosis factor α (rTNF-α) or macrophages. Resistance is accompanied by an increased dissociation constant for rTNF-α binding to high-affinity receptors on the HER2-transformed NIH 3T3 cells. The resistance phenotype is independent of transformation since NIH 3T3 cells transformed by the activated human homologue of the Harvey-ras oncogene (*HRAS*) retain high-affinity binding sites for rTNF-α as well as sensitivity to its cytotoxic effects. These results suggest that HER2 may potentiate tumorigenesis by inducing tumor cell resistance to host defense mechanisms.

Neoplastic cells undergo a number of heritable genetic alterations during the course of tumor progression *in vivo* (1–6). Subsequent to the initial event(s) leading to transformation, a subpopulation of tumor cells must survive selection by host immune surveillance to successfully establish a primary tumor. The role of oncogenes in transformation has been studied extensively (7), but the question of how transformed cells escape immune surveillance and form the primary lesion has been more difficult to approach. This latter step presumably involves the development of tumor cell resistance to a number of host defense mechanisms (3, 5, 6).

Tumor necrosis factor α (TNF-α) is a multifunctional protein (8, 9) that has been shown to play an important role in tumor cell killing *in vitro* by activated macrophages (10–12) and natural cytotoxic cells (13–15). TNF-α is also produced by a number of other immune cells thought to have a role in antitumor activity *in vivo* (8). Tumor cell resistance to recombinant TNF-α (rTNF-α) has been shown to correlate with resistance to cytotoxicity induced by macrophages (10) and natural cytotoxic cells (15). Little is known about mechanisms leading to tumor cell resistance to rTNF-α, although cellular growth factors have been implicated (16). In the work described here we have investigated the role of the oncogene that has been designated *ERBB2*; we have called this oncogene and its product HER2 for human epidermal growth factor receptor 2. It has also been called *NGL* and human c-*erbB*-2. The HER2 oncogene encodes a tyrosine kinase receptor-like protein

(p185^HER2^) with homology to the epidermal growth factor receptor [EGFR; also called ERBB1 and HER1 (17, 18)] in the induction of resistance to rTNF-α. We chose this oncogene for study because: (*i*) Recent work has shown that overexpression of unaltered HER2 oncogene coding sequences results in transformation and tumorigenicity of NIH 3T3 cells (19, 20); (*ii*) amplified HER2 oncogene expression occurs in many breast tumor cell lines (18, 20–22); and (*iii*) recent epidemiologic studies have found that amplification of HER2 is often associated with aggressive human breast cancers (23–25). The results reported here demonstrate that amplification of HER2 oncogene expression induces resistance of NIH 3T3 cells to the growth inhibitory effects of rTNF-α and activated macrophages *in vitro*. Induction of resistance to rTNF-α in this model system is accompanied by alterations in the binding of rTNF-α to its receptor. This work suggests a novel mechanism, induction of tumor cell resistance to rTNF-α, by which certain oncogenes may potentiate tumor formation.

MATERIALS AND METHODS

Cell Culture. Parental, nontransformed, NIH 3T3 cells were obtained from A. Levinson (Genentech) and strictly maintained in subconfluent culture as described (19). NIH 3T3 cells grown under these conditions demonstrate sensitivity to recombinant human TNF-α comparable to that of the murine L-M fibroblasts used for the standard TNF-α cytotoxicity assay (26). The control cell line (NIH 3T3 neo/dhfr, in which neo refers to the bacterial gene for neomycin phosphotransferase and dhfr refers to the mouse gene for dihydrofolate reductase), and cell lines expressing amplified levels of HER2 were prepared as described (19). The primary HER2 gene transfectants (designated HER2-3 cell lines) do not have a transformed morphology and fail to grow in soft agar (19). Stepwise amplification of HER2 gene expression by sequential selection of HER2-3 cells for growth in 200 nM, 400 nM, and 800 nM methotrexate gave rise to the cell lines HER2-3$_{200}$, HER2-3$_{400}$, and HER2-3$_{800}$. These cell lines show an increased ability to grow in soft agar and to form tumors in nude mice (19). The cell line NIH 3T3 neo/dhfr$_{400}$ was derived from NIH 3T3 neo/dhfr by subculture in 400 nM methotrexate. NIH 3T3 cells transformed by activated human *HRAS* (cell lines H-ras$_1$, H-ras$_2$, and H-ras$_3$) were prepared as described by Capon *et al.* (27).

Breast tumor cell lines were obtained from the American Type Culture Collection and were maintained in Ham's F-12 medium/Dulbecco's modified Eagle's medium, 1:1 (vol/vol), supplemented with 10% heat-inactivated fetal bovine serum,

Abbreviations: r, recombinant; TNF-α, tumor necrosis factor α; rTNF-α, recombinant TNF-α; HER2, human epidermal growth factor receptor 2 (also known as c-erbB-2); HER1, human epidermal growth factor receptor 1; neo, neomycin phosphotransferase; dhfr, dihydrofolate reductase.

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100 units of penicillin per ml, and 100 μg of streptomycin per ml. rTNF-α-resistant NIH 3T3 fibroblasts (NIH 3T3 neo/dhfr HTR) were derived by selection of NIH 3T3 neo/dhfr in medium containing 10,000 units of rTNF-α per ml. NIH 3T3 neo/dhfr HTR were maintained identically to parental NIH 3T3 cells except that rTNF-α at 10,000 units/ml was continuously present in the culture medium.

Immunoprecipitations and Labeling. For labeling experiments, 1.0 × 10⁶ cells were plated in 60-mm culture dishes (Falcon) and allowed to adhere for 12 hr. Metabolic labeling was done by adding 200 μCi of [³⁵S]methionine (Amersham, 1132 Ci/mmol; 1 Ci = 37 GBq) to 1.5 ml of methionine-free labeling medium containing 2% dialyzed fetal bovine serum. After 8 hr the cells were lysed and the HER2 oncogene-encoded p185, designated p185^{HER2}, was analyzed as described (19). Autophosphorylation assays of p185^{HER2} were as described (19). Immunoprecipitations of p185^{HER2} from metabolically labeled cells or for autophosphorylation assays were done by using a rabbit polyclonal antibody raised against a synthetic peptide derived from the carboxyl-terminal 17 amino acids of p185^{HER2} (17, 19).

Cytotoxicity Assays. Human rTNF-α was produced in *Escherichia coli* and purified as described (28). Cytotoxicity assays using rTNF-α (5.0 × 10⁷ units/mg of protein) (26) were performed as described by Lewis *et al.* (29). Macrophage cytotoxicity assays were done by the 72-hr ⁵¹Cr-postlabeling assay (10), except that activated human blood monocytes were used as effector cells. Blood monocytes were obtained by adherence to plastic for 1 hr at 37°C. Adherent cells were scraped and resuspended in medium, activated for 4 hr with 10 μg of *E. coli*-derived lipopolysaccharide (Sigma) per ml and 100 units of recombinant human γ interferon (2.0 × 10⁶ units/mg; Genentech). After activation the monocytes were washed and added to target cells at the appropriate effector:target ratios.

Receptor Binding. Receptor binding assays were performed as described (30, 31). Scatchard analysis of the receptor binding data was performed with the aid of the LIGAND program (Peter Munson, National Institutes of Health).

RESULTS

Analysis of the HER2-3 primary transfectant and cell lines derived from it by selection in methotrexate showed that expression of p185^{HER2}, as determined by metabolic labeling (Fig. 1*a*) and measurement of the associated tyrosine kinase (Fig. 1*b*), increased in parallel during amplification. Quantitative densitometry of the *in vitro* autophosphorylation reactions showed that the tyrosine kinase activity increased at least 5- to 6-fold between HER2-3 and HER2-3$_{200}$ and between HER2-3$_{200}$ and HER2-3$_{400}$, whereas only a small difference in activity was observed between HER2-3$_{400}$ and HER2-3$_{800}$ (Table 1).

The cell lines described above were tested for sensitivity to rTNF-α and macrophage-induced cytotoxicity. Stepwise amplification of p185^{HER2} expression resulted in a parallel induction of resistance to rTNF-α (Fig. 2*a* and Table 1). The primary transfectants (HER2-3), which do not have a transformed phenotype (19), demonstrated a slight increase in resistance at low rTNF-α concentrations (Fig. 2*a*), but no difference in resistance was observed at a rTNF-α concentration of 10,000 units/ml (Fig. 2*a* and Table 1). However, the transformed lines HER2-3$_{200}$, HER2-3$_{400}$, and HER2-3$_{800}$ did show a stepwise loss in sensitivity to rTNF-α-mediated cytotoxicity as compared with NIH 3T3 neo/dhfr cells at all TNF-α concentrations tested (Fig. 2*a* and Table 1). The difference in sensitivity of HER2-3$_{200}$ and HER2-3$_{400}$ (27.5% versus 48.4% viability at 10,000 units of rTNF-α per ml) was
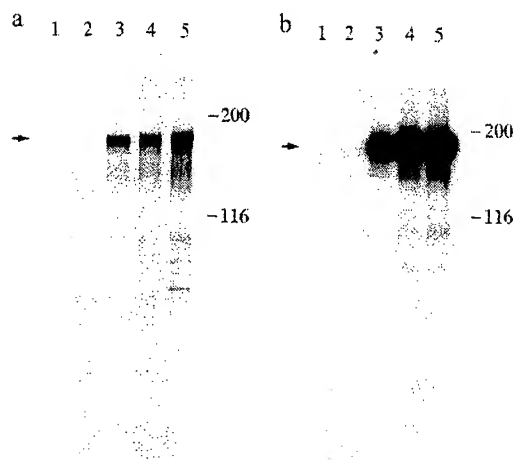

Fig. 1. Amplification of HER2 oncogene expression. Autoradiographs are shown of [³⁵S]methionine-labeled p185^{HER2} (*a*) and autophosphorylated p185^{HER2}-associated tyrosine kinase (*b*). Labeling and processing of samples were done as described in *Materials and Methods* and ref. 19. Sizes are shown in kDa. Lanes: 1, NIH 3T3 neo/dhfr cell line; 2, HER2-3; 3, HER2-3$_{200}$; 4, HER2-3$_{400}$; 5, HER2-3$_{800}$.

greater than the difference between HER2-3$_{400}$ and HER2-3$_{800}$ (48.4% versus 58.7% viability; Table 1). A similar result was obtained when NIH 3T3 neo/dhfr and HER2-3$_{800}$ were compared for sensitivity to activated macrophages (Fig. 2*b*), although direct selection for resistance to rTNF-α resulted in a cell type (Fig. 2*b*; NIH 3T3 neo/dhfr HTR) that displayed more resistance to macrophage-induced cytotoxicity than was obtained by overexpression of HER2. Similar results, which suggest that rTNF-α-resistant cells are also resistant to activated macrophages, have been reported (10, 15). The mechanism of resistance to rTNF-α in NIH 3T3 neo/dhfr HTR is unknown but does not appear to involve the autocrine production of TNF-α (data not shown). In addition, selection

Table 1. Relationship between HER2-associated tyrosine kinase levels and resistance to rTNF-α

Cell type	% viability	Relative tyrosine kinase
NIH 3T3 neo/dhfr	3.6 ± 0.6	*
NIH 3T3 neo/dhfr$_{400}$	8.3 ± 1.0	*
HER2-3	2.0 ± 0.4	1.0
HER2-3$_{200}$	27.5 ± 2.7	6.7
HER2-3$_{400}$	48.4 ± 1.4	32.5
HER2-3$_{800}$	58.7 ± 1.3	39.6
MCF7	2.5 ± 0.3	1.0
BT-20	1.6 ± 0.3	<0.4
MDA-MB-231	64.2 ± 9.3	<0.4
MDA-MB-175-VII	31.2 ± 4.4	3.5
MDA-MB-361	26.8 ± 6.6	41.0
SK-BR-3	56.4 ± 5.5	119.2

Percent viability is given at 10,000 cytotoxicity units of rTNF-α per ml (26). Tyrosine kinase assays were performed as described (19). Relative amounts of tyrosine kinase present in each of these cell types were determined by taking ratios of the areas under the curves obtained by scanning autoradiograms (with an LKB2202 laser densitometer). The autoradiograms had been exposed for various times to allow for linearity in the determinations and then normalized by comparison to the HER2 gene primary transfectant (HER2-3) for the NIH 3T3-derived cell lines and to MCF7 for the human breast tumor cell lines. The derivation of the NIH 3T3 cell lines is described in text.
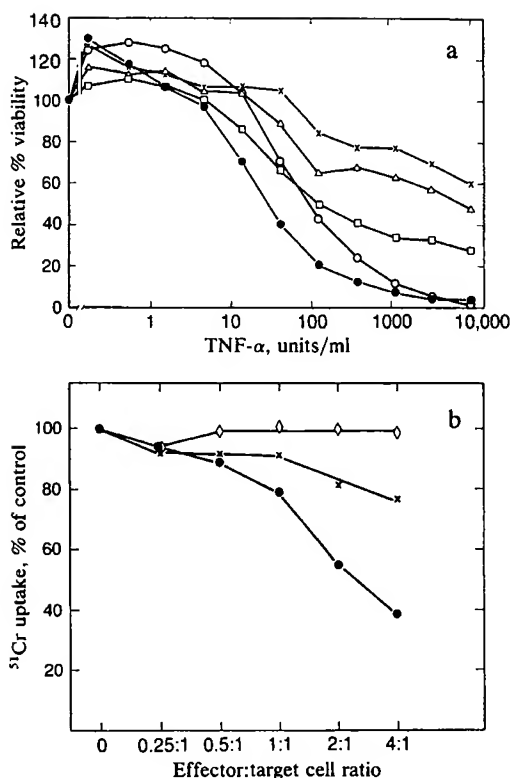*Not measured.

FIG. 2. Induction of resistance to rTNF-α and macrophages by amplified expression of HER2 oncogene. (*a*) rTNF-α resistance of control and HER2 gene-transfected NIH 3T3 cells. (*b*) HER2 induction of cellular resistance to cytotoxicity mediated by activated macrophages. Each assay was done at least six times. The coefficient of variance of the data was <10%. Cell lines: ●, NIH 3T3 neo/dhfr; ○, HER2-3; □, HER2-3$_{200}$; △, HER2-3$_{400}$; ×, HER2-3$_{800}$; ◇ in *b*, NIH 3T3 neo/dhfr HTR.

of the control cell line, NIH 3T3 neo/dhfr, for resistance to 400 nM methotrexate, resulting in cell line NIH 3T3 neo/dhfr$_{400}$, did not result in altered resistance to rTNF-α (Table 1). This result suggests that it was amplified expression of the HER2 oncogene and not the associated drug-resistance markers (*neo*, *Dhfr*) that conferred rTNF-α resistance to NIH 3T3 cells.

The observation that NIH 3T3 cell lines expressing high levels of p185^{HER2} were resistant to cytotoxicity induced by rTNF-α or macrophages, coupled with previous observations associating HER2 gene amplification with aggressive breast cancer (23, 24), suggested that this may be one mechanism leading to breast tumor development. To test this possibility we screened six breast tumor cell lines for expression of p185^{HER2}-associated tyrosine kinase activity and for sensitivity to TNF-α-mediated cytotoxicity. The results (Table 1) showed that, of three breast tumor cell lines with no amplified expression of HER2 oncogene, two were sensitive to growth inhibition by rTNF-α (MCF7 and BT-20), while one was resistant (MDA-MB-231). However, of the cell lines demonstrating increased expression of p185^{HER2} (MDA-MB-175-VII, MDA-MB-361, and SK-BR-3), all three were resistant to growth inhibition by rTNF-α. These results suggest that amplified expression of p185^{HER2} is associated with resistance to rTNF-α, but that there must be alternative mechanisms of resistance to rTNF-α because one of the rTNF-α-resistant tumor cell lines (MDA-MB-231) did not show amplified expression of the HER2 oncogene.

We have also explored whether NIH 3T3 cell resistance to rTNF-α may occur as a result of transformation by other

oncogenes. To accomplish this, NIH 3T3 cells were transfected by the activated *HRAS* oncogene (27). Three independent transformants were selected (H-ras$_1$, H-ras$_2$, and H-ras$_3$) and tested for resistance to rTNF-α. The viability of these cell lines in the presence of 10,000 units of rTNF-α per ml (H-ras$_1$, 13.3 ± 0.8%; H-ras$_2$, 6.6 ± 0.5%; H-ras$_3$, 9.9 ± 1.1%) was comparable to the nontransformed NIH 3T3 neo/dhfr cell line (Table 1).

The mechanism by which transformation by the HER2 oncogene can induce resistance to rTNF-α in these cells is unknown. One possible explanation could be direct or indirect receptor transmodulation (32). Down-regulation of the activity of the receptors for epidermal growth factor by activation of the platelet-derived growth factor receptor or phorbol esters has been documented (33–41). The possibility that TNF-α receptor function may be similarly down-regulated was suggested by recent work demonstrating that pretreatment of tumor cells by phorbol esters induced resistance to macrophage-mediated cytotoxicity (42), probably due to down-regulation of TNF-α receptors (43, 44). To investigate whether the TNF-α receptor was altered in HER2-3$_{800}$ cells as opposed to NIH 3T3 neo/dhfr cells, the binding of ^{125}I-labeled rTNF-α (^{125}I-rTNF-α) was compared between these cell lines. The results showed a 2- to 3-fold increase in total specific binding for HER2-3$_{800}$ as compared with NIH 3T3 neo/dhfr (Fig. 3*a*). In addition, the displacement curve for binding of ^{125}I-rTNF-α on HER2-3$_{800}$ cells is shifted toward lower affinity binding as compared with NIH 3T3 neo/dhfr cells. Scatchard analysis of the competition binding data indicated that NIH 3T3 neo/dhfr cells (Fig. 3*c*) demonstrated high-affinity binding of rTNF-α ($K_d = 1 \times 10^{-10}$ M) similar to that reported for other cell lines (45–48). However, the high-affinity binding of rTNF-α on HER2-3$_{800}$ (Fig. 3*d*) was lower by a factor of 20 ($K_d = 2 \times 10^{-9}$ M). Both cell types possessed large numbers of low-affinity TNF-α receptors ($K_d \cong 1 \times 10^{-7}$ M), as has been reported (31) for 3T3 L1 Swiss mouse embryo fibroblasts. In contrast to these results, cellular transformation by the activated *HRAS* oncogene has no effect on TNF-α receptor number or affinity as compared with the NIH 3T3 neo/dhfr control cell line (Fig. 3*b*, *e–f*).

DISCUSSION

Tumor cell cytotoxicity may be mediated by a variety of mechanisms depending upon the tumor and effector cell types (for review see ref. 49). TNF-α is one of a group of cytokines and other mediators of tumor cell cytotoxicity. Other cytokines that show cytotoxic activity alone and that may have enhanced activity in the presence of rTNF-α include γ interferon (45) and interleukin 1 (50, 51). Natural killer cells and cytotoxic T cells may elaborate TNF-α or a similar molecule but clearly can induce tumor cell cytotoxicity by TNF-α-independent mechanisms (52, 53). While the relative significance of each of these factors or combination of factors still remains to be fully evaluated, emerging evidence strongly supports the role of the macrophage in the early stages of antitumor surveillance (6) and also the role of TNF-α as an important effector molecule in macrophage-mediated tumor cell cytotoxicity (10–12).

Our results show that increasing levels of expression of p185^{HER2} in NIH 3T3 cells leads to induction of resistance to cytotoxicity induced by rTNF-α. As has been reported in other model systems (10), induction of resistance to rTNF-α is accompanied by increased resistance to macrophage-induced cytotoxicity. Our observation that NIH 3T3 fibroblasts selected directly for resistance to rTNF-α (NIH 3T3 neo/dhfr HTR) are more resistant to activated macrophages than the HER2-3$_{800}$ cell line suggests that mechanisms other than amplified expression of oncogenes may lead to resistance when the direct selection protocol is used (54). Alternatively, direct exposure of a cell population to rTNF-α could
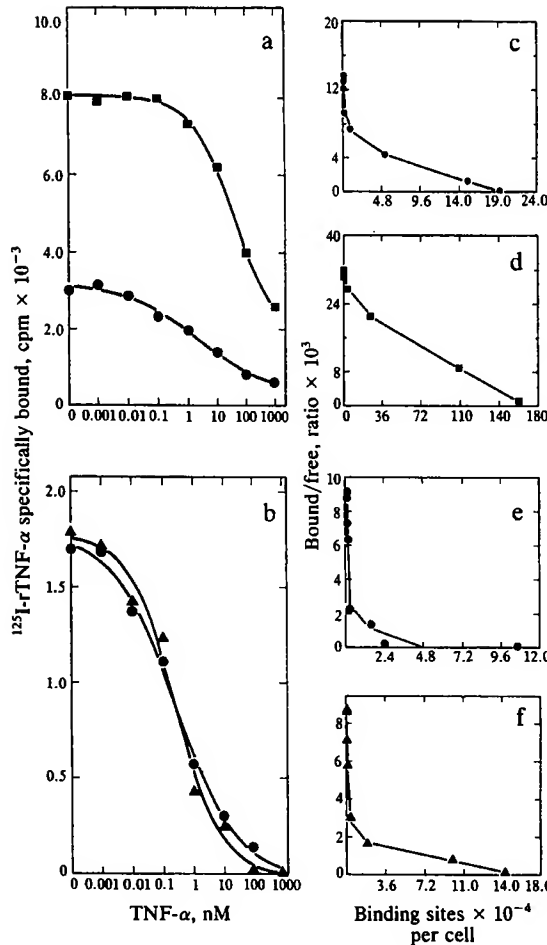
rather that the product of the HER2 gene in particular may directly or indirectly affect TNF-α receptor function. Similar receptor transmodulation has been observed in other growth factor systems (33–41).

Gene amplification associated with drug resistance has been documented in many systems (55), but this report associates amplification of oncogene expression with the dampening of the response of a tumor cell to a well-defined mediator of macrophage-induced tumor cell cytotoxicity. These results suggest that amplified expression of the HER2 oncogene not only may induce cellular transformation (19, 20) but also may play a role in tumor progression by limiting the efficacy of host defenses against neoplasia.

FIG. 3. TNF-α receptor binding analysis. (*a* and *b*) Displacement curves of ^{125}I-rTNF-α binding to cell lines NIH 3T3 neo/dhfr (●) and HER2-3$_{800}$ (■) in *a* and to NIH 3T3 neo/dhfr (●) and H-ras (▲) in *b*. (*c* and *d*) Scatchard analysis of the binding data in *a*. (*e* and *f*) Scatchard analysis of the binding data shown in *b*. The binding experiments were done three times with similar results. Data analysis was carried out as described in *Materials and Methods* and ref. 44.

result in selection of a subpopulation of tumor cells overexpressing as yet unidentified oncogenes which are more potent in their induction of rTNF-α resistance than is HER2. The possibility that mechanisms other than amplification of HER2 oncogene expression may lead to rTNF-α resistance is reinforced by our data indicating that of the six breast tumor cell lines examined, one was resistant to the cytotoxic effects of this monokine but did not show detectable expression of the p185^{HER2}-associated tyrosine kinase.

Receptor binding analysis comparing NIH 3T3 neo/dhfr and HER2-3$_{800}$ cell lines suggests a possible mechanism by which the HER2 oncogene may induce rTNF-α resistance. The high-affinity TNF-α receptors of the former cell line demonstrate a K_d of 1×10^{-10} M for TNF-α, which is comparable to that reported for other cell lines (45–48), but the TNF-α receptors of HER2-3$_{800}$ cells have an affinity reduced by a factor of 20 ($K_d = 20 \times 10^{-9}$ M) for ligand binding. It may be that the elimination of this high-affinity class of receptors leads to reduction of the cytotoxic response. Our finding that transformation by the activated *HRAS* oncogene does not affect rTNF-α cytotoxicity or receptor function on NIH 3T3 cells suggests that induction of resistance is not a general property of transformation, but

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